Building a two-photon microscope is easy

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Building a two-photon microscope is easy because most of the work is done by the laser itself. All the microscope needs to do is to focus the laser light to a point, move it across the preparation, and measure the fluorescence photons emitted. These jobs are done by an objective, a scan engine, and a detector, respectively. That's all there is to it.

1 Introduction

1.1 Fluorescence

When we look around the world, our eyes are usually detecting photons that originated with a familiar light source (e.g., the sun, or a light bulb), and bounced off and through objects eventually reaching our eyes. However, when we view fluorescence (e.g., fluorescent paint in a blacklight poster) the fluorescence photons that reach our eyes did not originate with the light source. Instead, the fluorescent molecules absorbed photons from the light source and then emitted different photons.

We can illustrate the process in a Jablonski diagram, which illustrates the energy levels of a molecule. In the case of green fluorescent protein [1], a blue photon is absorbed, and the absorbed energy sends the molecule into an excited state. A small fraction of the energy is dissipated through heat, and then the molecule relaxes back down to the ground state, emitting a green photon in the process (Fig 1a). The entire process of absorption and emission takes a few nanoseconds, and most of that is on the emission side.

1.2 Two-photon absorption

In two-photon excitation, instead of a single high photon being absorbed, two lower energy infrared photons are absorbed. This is disallowed in classical physics, because each absorption is a single event. If the photon has insufficient energy to get the molecule to the excited state, then the molecule simply does not make the transition. Multiple absorptions do not help in the classical case. For example, to jump a six-foot fence, a six-foot leap is required—two three-foot leaps are insufficient. However, in quantum mechanics, there is a level of uncertainty, formalized by Heisenberg's Uncertainty Principle [2]. This uncertainty permits two low-energy photons to excite a molecule, provided that they arrive at nearly the same place, at nearly the same time.

To illustrate two-photon absorption in a Jablonski diagram, scientists often draw a "virtual state" between the ground state and the first excited state, as if there is an intermediate energy level (Fig. 1b). This is useful for explaining the concept of two-photon excitation, but it is incorrect to consider the virtual state this way, because the virtual state is not a well-defined energy level. In fact, Maria Göppert-Mayer drew the virtual state *above* the first excited state in the Jablonski diagrams in her thesis [3]. The two photons absorbed do not have to be of the same wavelength. Non-degenerate (i.e., two photons of different wavelengths) two-photon excitation has been explored for microscopy applications [4].

2 Materials & Methods

2.1 The laser

The laser is the workhorse of the system, and the first two-photon microscope [5] was developed following improvements in laser technology [6,7]. Two-photon absorption is a highly unlikely event under bright sunlight (~ once every 10 million years) [8]. For two-photon excitation, the incident light must have an intensity many orders of magnitude higher. There must be so many photons that the molecule absorbing them can't—quantum-mechanically speaking—tell where one photon ends and the next one begins [3]. Thus, extremely high light intensities are required to obtain sufficient rates of fluorescence for imaging. These high intensities can be generated in the brief pulses of light from ultrafast lasers.

The likelihood of a two-photon excitation event for a given molecule is measured by its two-photon cross-section, which is a property of the fluorescent molecule and the wavelengths of the exciting photons. The cross-section is measured in units of cm^4s, which may seem like unusual units at first, but are trivial to derive. Start with a more familiar, macroscopic cross-sectional area, which can be measured in cm^2. The two-photon cross-section is the product of two of these conventional cross-sections (each measured in cm^2), and the lifetime of the virtual state (measured in seconds). Typical one-photon cross sections for fluorescent molecules are approximately 10^{-17} cm^2 and virtual state lifetimes are approximately 10^{-15} s. Thus, two-photon cross-sections are on the order of 10^{-49} cm^4s. For convenience, a special unit is used, and for respect, it is named after Maria Göppert-Mayer: 10 Göppert-Mayer units, or 10 GM = 10^{-49} cm^4s. Fluorescent dyes and proteins that are commonly used in two-photon neuroimaging have cross-sections from about 10 to 300 GM.

Ultrafast lasers used for two-photon excitation emit incredibly intense pulses of light. Today, the most commonly used ultrafast laser used in two-photon imaging is the titanium-doped sapphire (Ti:Sapph) laser [9]. These systems generate average powers of about 1 – 10 watts. However, they emit few if any photons 99.999% of the time. All the energy they produce is squeezed into ~100 fs pulses, and these are emitted at rate of ~80 million times per second. It is difficult to grasp how brief 100 fs is, so let's scale it up for comparison. If a 100 fs long pulse would be scaled up to one second, then there would be about a day and a half between adjacent one-second-long pulses. Thus, with these parameters we have both high peak powers to ensure two-photon excitation events, and also moderate average power at the sample (~0.01 – 0.1 watts) to minimize tissue heating and damage [10-12].

Ti:Sapph laser systems are convenient due to their tunability (~700 – 1000 nm), and these remain the most popular technology for two-photon microscopy. However, they are relatively complex and expensive,

typically making up the bulk of the system cost. Three-photon imaging requires wavelengths (e.g., 1300 nm) out of the range of conventional Ti:Sapph systems, and thus optical parametric amplifiers are used [13]. Laser technology is a rapidly developing area, and new options are emerging, including fiber lasers [14].

The laser is the most critical component in a two-photon imaging system, and where our system diagram starts (Fig. 2). Next, a scan engine will move the beam around the specimen, and an objective will focus the beam to a small volume in the sample. Finally, a collection system will direct the emitted photons to a detector (usually a photomultiplier tube, PMT). Those are the parts we will discuss in the following sections. Note that most of these optics, in their basic configurations, are quite simple. Galileo Galilei would recognize many of these optical systems.

2.2 The scan engine

The scan engine takes the laser beam and angles it into the objective so that it excites different regions of the sample. The beam width extends across the entire back aperture of the objective, and it's center should not move. When the beam approaches the back aperture of the objective along the central optical axis, the center of the focal plane is excited. When the beam hits the back aperture at an angle, a lateral point in the focal plane is excited. Thus, the job of the scan engine is to rapidly alter the angle of approach of the beam (Fig. 2).

One might not even need a scan engine if the sample can be moved fast enough. Instead, one could leave the beam fixed in space and simply move the sample to image it. However, this is often not an option for biological samples, and even if it were, it is unlikely that the sample could be moved rapidly enough to provide fast, high quality sampling that is free from movement artifacts. In particular, many *in vivo* preparations would not appreciate such jostling. Thus, we usually opt to scan the beam and leave the sample stationary.

A great deal of creativity and optimization can be exercised in the design of the scan engine, perhaps more so than any other component of a two-photon imaging system. Let us start with the basics. Usually we are using an infinity-corrected objective, so we need to provide collimated light to the back focal plane of the objective and vary the angle of approach (the angle between the optical axes of the laser beam and the objective) to translate the excitation volume across the sample.

The most conventional way to do so is to use scanning mirrors. The deflection angle of a scan mirror can be relayed to the back aperture of the objective using a scan lens and tube lens in series [15]. Scan mirrors typically scan in one axis only (X or Y), and thus two are placed in series to provide access to the full field-of-view of a system. Ideally, the scan mirrors should be optically relayed to one another using lenses or mirrors [16-18]. However, if only the central field-of-view is needed, the scan mirrors may be placed very close together, provided that the inter-mirror spacing is much smaller than the focal length of the scan lens [15].

Galvanometer-based scan mirrors provide relatively fast scanning and can respond to arbitrary command waveforms within their operating envelope, with smaller mirrors offering faster response times. Resonant scanners can operate about four to ten times faster than galvanometer-based scanners. However, that speed comes at a price. The scan amplitude can be adjusted, but that's it. The scan speed cannot be changed. Unfortunately, the sinusoidal scan pattern is fastest (in degrees per second, or microns scanned per second) in the middle of the field-of-view and slowest at the edges—exactly the opposite of what would be ideal for imaging [19]. Linear scanners are usually set to scan the field-of-view at a constant rate and then speed up at the edges to get ready for the next line. Linear scanners also use a variable scan offset to "pan" around the field-of-view. Resonant scanners cannot be used to "pan" in this way, the scanned region will always be centered in the same place, in the center of the resonant axis. To address this limitation, another linear scanner can be added (e.g., resonant X $\rightarrow$ linear X $\rightarrow$ linear Y), and this offers great flexibility, at the cost of increased complexity.

It is also possible to add on rapid Z-scanning abilities via tunable lenses [20], piezo-actuated objective positioners [21], or adaptive optics [22,23]. Tunable lenses are the simplest (and least expensive) to implement, but they add aberrations that impair imaging when focusing far from the objective's natural imaging depth. Piezo-actuated objective positioners are more expensive, and involve considerable motion next to the sample, but can offer lower aberration imaging. Adaptive optics offer low aberration imaging, fast response times, and no objective motion. However, they are the most expensive and involved of the three options mentioned above. Rapid scanning can also be supported by spinning mirrors [24], acousto-optical deflectors [25-27], and ultrasound lenses [28]. Moreover, spatial light modulators and holographic techniques can be used to reposition the excitation as well [29-31]. Pushing the limits of scan engine designs often requires careful optical design to minimize optical aberrations [18,32]. Indeed, scan engines can quickly become rather complex, and we have only touched on the variety here [33]. Moreover, the scan pattern itself can be optimized for a particular measurement. For example, instead of raster scanning an entire image plane, the beam could be directed along an arbitrary path to sample from the key regions of interest within the field-of-view. The desired scan pattern can influence the scan engine design in turn. Scan engine design is certainly a rich area for creative optical engineering.

2.3 The objective

Two-photon excitation works best with high numerical aperture (NA) objectives for two reasons: the excitation photons are concentrated to a higher intensity, which increases the likelihood of getting excitation events (and resulting fluorescence photons), and high NA systems will collect a large fraction of emitted fluorescence photons.

What you just read isn't completely true in practice. When imaging the brain, the scattering of the tissue degrades the performance of high NA systems more severely than more moderate NA systems [34,35]. This is partly because the marginal rays for high NA systems have so great a path length through the tissue. Along these greater path lengths, there are more opportunities for scattering and absorption events. Moreover, when imaging hundreds of microns into a sample (where the strength of two-photon excitation is key), tissue-induced aberrations reduce the effectiveness of high NA focusing [36]. Therefore, while higher NA is always better for signal collection, in practice, moderate NA (0.40 – 0.80) systems can be sufficient for two-photon imaging [18,32,37]. Moreover, moderate NA systems can offer ergonomic benefits including longer working distances, more flexibility on immersion media (e.g., air objectives, which often have lower NA than water-dipping objectives), and access angle to position instruments including electrodes within the field-of-view of the objective.

High NA objectives are sensitive to many parameters that are not appropriately constrained in many neurobiology experiments. For example, high NA objectives (> 1.0 NA) are designed to be used at a specific temperature, and their performance can drop precipitously over just a few degrees Celsius from their design temperature. Also, high NA objectives are designed to be used with a particular type of coverslip, but some neurobiological preparations use no coverslip while others use stacks of three coverslips on top of each other (few objectives are designed for such use). Consult with the manufacturers to determine what imaging conditions their nominal performance is specified for. The good news is that high NA objectives can still be used in non-ideal circumstances. Even if they are not offering their nominal optical performance, their residual performance can exceed that of moderate NA systems. Relatedly, objectives of any NA must be overfilled at their back aperture to use all of their excitation NA and offer the best possible resolution [38]. However, underfilling objectives, particularly high NA objectives, can still yield acceptable results.

The NA governs the resolution of the system, among other factors [39], and the required resolution varies by application. Sometimes relatively small excitation volumes (related to the point spread function, PSF, [40]) are required, while in other applications this requirement can be relaxed, sometimes deliberately so. In calcium imaging, several techniques have been used to engineer expanded PSF for specific applications. Extending the excitation volume axially [41-43], can provide faster scanning of a volume since fewer imaging planes are scanned, at the cost of reduced axial resolution. For sparsely labeled samples, that trade-off is often acceptable because there are fewer structures above and below the region of interest that yield contaminating signals. Axially extending the excitation volume can also help make imaging less sensitive to axial shifts which can occur during movement of the preparation or session-to-session misalignments. Small transverse drifts or misalignments can often be corrected offline because the data is there, just in a different pixel location. However, with high resolution optical sectioning, axial shifts cannot be corrected for (unless multiple Z planes are imaged at each time point). Extending the excitation volume laterally, with temporal focusing [44] can support faster scanning, as long as the tradeoff in spatial resolution is tolerable.

For most applications, water dipping objectives are preferred. These systems can provide good optical performance, and facilitate the simultaneous use of glass micropipettes for visualized patch clamp recording. Air objectives can offer ergonomic benefits in some situations. When working with objectives positioned at large angles (off of vertical), or with preparations that are rapidly switched in and out (e.g., automated head-fixing systems [45,46]), not needing immersion media can simplify the instrumentation significantly. A drawback to air objectives is the relatively large mismatch in index of refraction, *n*, between the glass of the objective and air, and between air and the physiological sample. This leads to spherical aberration and alters Z measurements [47]. These aberrations can be minimized with moderate NA objectives (aberration effects are greater with higher NA systems) and compensated for with adaptive optics.

Objectives have several parameters that should be optimized for two-photon imaging. They should, of course, have appropriate anti-reflective (AR) coatings. The scan engine needs appropriate AR coatings as well, but only for the infrared excitation wavelengths. The objective must pass visible wavelengths well, with the infrared wavelengths a close second priority. Transmission rates over 80% are typical for objectives across the visible and infrared range. Objectives should also be relatively achromatic across the infrared wavelengths used to minimize pulse distortions[48], and these can be determined using standard raytracing software during design or modeling [49]. However, obtaining such specifications from manufacturers can be difficult if not impossible. The simplest approaches are to test a candidate objective on a working two-photon imaging system, or to use commercial objectives that have already been proven to work well for two-photon imaging. When testing optics, be sure to test on a specimen that is similar or identical to the specimens on which measurements will be made.

Some samples, like pollen grains, can be very forgiving and provide excellent looking images when actual system performance is far from ideal.

Again, we are only scratching the surface of the parameter space that can be explored. Most two-photon microscopes use off-the-shelf components, especially the objective. In those cases, the rest of the system is built around those off-the-shelf components. An alternative approach is to start completely from scratch and design end-to-end custom optics, optimized for a specific application [17,50]. This approach affords even greater creativity.

2.4 The detector

Collection optics gather fluorescence photons from the objective and direct them to a detector. Theoretically, it can be difficult to design an optimal collection system, in part because scattered light cannot be focused, by definition. Large diameter collection optics can be helpful in collecting scattered light [51,52]. Simple two-lens schemes function well in many setups [53], and in practice, the position tolerance of the collection is often not tight (i.e., translating individual lenses +/- a few millimeters along the optical axis has minimal effects on signal collection in some systems). Again here, there is room for great creativity in the design of these optics, including supplemental collection [54], oil immersion optics [17], and coupling to liquid light guides [55].

The detector should be several square millimeters in area, have a high bandwidth (~1 GHz), high quantum efficiency, and low noise. Multi-alkali PMTs, GaAsP PMTs, and hybrid photodetectors [56] offer this combination of features. These devices, which are all single pixel detectors, may seem like a slow way to image, but it's actually the ideal solution for multiphoton imaging deep in scattering tissue. Fluorescence photons should only be created within the excitation volume. Some of these photons will make it out of the tissue without being scattered, and these are called ballistic photons, and they not only reflect signal intensity, but also can be used to determine where in the tissue the fluorescence emission occurred (based on the location and angle at which the photon hits the detector). However, the single pixel detector discards the spatial information and only records the presence of the photon. The spatial information is redundant, because the scan engine already determines where the signal could come from. Other fluorescence photons will be scattered one or more times before exiting the tissue, but they can still make it to the detector. These photons can contribute to overall signal intensity, but the angle and location at which they hit the detector cannot be used to determine where in the tissue the fluorescence emission occurred. Again, this is fine because the scan engine is controlling the location of the excitation volume, so spatial information from the fluorescence photons is not needed. Instead, both ballistic and scattered photons contribute to signal intensity.

Area detectors, or cameras, can be used with two-photon imaging. However, because cameras have integration times (> 1 ms) that are much longer than typical pixel dwell times in two-photon imaging (< 1 µs), they will detect photons from multiple excitation locations during the same integration window. This can be acceptable when almost all of the photons are ballistic (not scattered), and it opens the door to multibeam scanning and other techniques to more rapidly scan excitation energy across the preparation, potentially improving the overall frame rate [57]. However, when there is significant scattering (e.g., ≥ 400 µm deep in mouse neocortex), the images become blurred. This is because cameras rely on ballistic photons to form a high-resolution image, and scattered photons arrive at the "wrong" pixel locations, and blur the image. Thus, the use of area detectors defeats one of the key advantages of two-photon imaging: resolving structures deep in scattering tissue. Systems that use a camera for detection can only be used when imaging at very shallow depths in tissue, similar to 1-photon approaches.

Digitization of signals from the detector generally fall into two categories: photon counting and analog integration. The former can be closer to optimal under some imaging regimes, including very dim samples. However, it requires high bandwidth signal processing, and some implementations sacrifice dynamic range. Analog integration is a more conventional approach and is sufficient for many experiments. Again, there are variations of these approaches including lock-in sampling (at a fixed delay from the pulses of the laser). The relative merits of these different approaches are the subject of much discussion [58] and offer yet more opportunities for creativity and interesting engineering trade-offs.

3 Methods

3.1 Design constraints

The design of any imaging system is constrained by engineering tradeoffs. A design is often trying to simultaneously maximize several competing parameters: frame rate, resolution, and field-of-view. Dynamics in neurobiology often play out at the subsecond time scale, with micrometer resolution, across millimeters to centimeters of neural circuitry. Systems cannot be designed to meet arbitrary specifications, and thus a good deal of the creativity in design comes from deciding which compromises to make.

A major limiting factor for the design of a two-photon imaging system is the expected yield of detected fluorescence photons per laser pulse. This is a function of the properties of the fluorescent indicator, and the laser power the sample can tolerate. Brighter samples and higher laser power will increase the photons per

pulse, of course. Too much laser power will cause damage to the preparation. These design factors, excitation power and signal photon levels, are sometimes called the “photon budget”.

Generally, the detected yield per laser pulse of photons from fluorescence is less than one. In a study by Driscoll and colleagues, their imaging system detected about ten fluorescence photons per pixel, when using 383 laser pulses per pixel, and thus an average of ~0.03 photons per excitation laser pulse [58]. This is a typical value for two-photon imaging in neuroscience. This is true even for the intense pulses used in three-photon imaging, as values from a functional imaging study show [13]. There were ~100 pixels per cell body in that study, and ~100 photons per second per cell. Converting that to photons/pulse, we get: 100 photons / (8.49 frames/s * 100 pixels per cell * 3 pulses per pixel) = 0.04 photons per pulse. Thus, across a range of imaging system parameters, the signal photons per laser pulse remain typically << 1.

This is important to keep in mind during system design. For example, when using a 12 kHz resonant scanner, each line is scanned in 42 µs (two lines per cycle). In that amount of time, an 80 MHz laser pulses 3360 times. If the line is split into 512 pixels, then there are < 10 pulses per pixel. Given the numbers above, there could be < 1 photon per pixel even in bright regions. Therefore, when scanning rapidly, temporal averaging is often necessary to clearly make out the image. There are a lot of pixel samples that are zero (or just noise).

Since two-photon imaging is a point-scanning technique, this low number of signal photons per laser pulse places a limit on the pixel acquisition rate (and by extension, the frame acquisition rate and/or frame size), given a minimal signal-to-noise specification. For example, given a particular resolution, as the field-of-view and/or depth (i.e., overall imaging volume) increases, the number of pixels per frame increases, and the time required to acquire a frame increases. Thus, it is often preferable to image portions of the full image and/or use multiple imaging beams to sample from multiple regions of an imaging volume [17,18,32,59-63].

Originally, two-photon imaging systems used mostly conventional microscope parts from widefield and confocal microscopes. These parts often suffice in practice, even though they were not designed specifically for two-photon microscopy. In recent years, the market options for components for two-photon imaging systems have expanded considerably. Today, for most systems, commercial off-the-shelf (COTS) components will be sufficient to perform the measurements needed for a particular experiment. For advanced applications, custom optics can be explored when COTS components cannot suffice [17,32,50,64]. Still, even custom optics are subject to the design constraints discussed above.

3.2 Software

Once the system is constructed, it must be connected up to a computer to synchronize beam scanning, signal digitization, image generation, live display, and other processes. Software for two-photon imaging typically grows in complexity as features are added, but the core essentials are simple. The computer needs to output scan mirror commands (e.g., sawtooth voltage commands, where the X frequency is N times the Y frequency, where N is the number of scan lines per imaging frame), digitize the detector signals, and construct the data stream into images. There is a lot of optimization and additional features that can be added, but these essentials can be coded quite compactly. Sophisticated image processing is not necessary for the data acquisition stage, particularly in the case of linear galvo scanning. In the case of resonant scanning, the speed of the fast axis varies nonlinearly across the field of view, and so the data needs to be processed to compensate for that aspect. The nonlinearity is predictable and relatively stationary, and thus the compensation not typically complex. Simple software can be developed in a day or two, sufficient for basic operation and testing. Subsequently, it typically grows into a labyrinthine suite as more functionality and features are added to support experiments.

4 Trouble shooting and further resources

4.1 Tips and tricks

Here are some brief practical tips building a two-photon microscope. This is to supplement the resources mentioned above.

“Imaging” the scan commands

When first setting up a new system, particularly when writing or customizing the software, it can be useful to “image” the scan commands. That is, run the analog command voltages for the X and Y mirrors into two acquisition channels. The channel corresponding to the X command should have a gradient from left to right, and the channel for the Y command should have a gradient from top to bottom. This quick spot check lets the operator troubleshoot the instrument control and image generation routines without having to actually image anything.

Aligning a two-photon imaging system

The individual lenses within a commercial objective lens are aligned by the manufacturer to high precision. The same goes for the individual lenses within a sophisticated scan lens. However, the relative spacing between the main components of a laser scanning microscope (e.g., scan lens, tube lens, objective) does not typically need to be highly precise—a difference of a few millimeters often makes little difference in performance. There is one exception: the distance

between the scan mirrors and the scan lens is critical. This distance has a relatively strong influence on how stable the beam will be at the objective, and the ultimate imaging quality. This distance needs to be carefully adjusted, and fortunately it is relatively easy to do so using visual feedback. First, lay out all of the scan optics with their designed spacings. Activate the scanning in software, at the maximum scan angle. Next, view the beam at the back focal plane of the objective and adjust the scan mirror-to-scan lens distance to make the beam relatively stationary during scanning. In addition, shearing interferometers are useful tools for measuring changes in beam quality as the beam passes through elements. Another trick is to let the beam project to a distant target and examine the beam shape at different points during scanning. For example, removing a mirror can let the beam travel several meters after the scan lens and hit a paper target on a laboratory wall. As the scan coordinates change, the beam shape should remain circular. Any distortions of the spot shape during scanning could indicate aberrations and/or clipping.

Imaging pollen grains and subresolution beads

The first target for a new imaging system should be a uniform fluorescent sample, for example a fluorescent piece of plastic or a tub of fluorescin. This provides an opportunity to fine tune the collection optics (maximizing the signal) and ensuring that excitation light is generally making it to the preparation, even if it the PSF is not yet optimized through fine alignment. After that step, it is time to focus on a sample with structure. Imaging a slide with pollen grains is handy, but remember that they are massively forgiving. This preparation is bright and sparse. It is easy to optimize a system using pollen grains and experience abject failure when imaging a living biological specimen. Use it only as a rough starting point. Then move to subresolution (smaller than the expected PSF) beads (preferably embedded in agar or some tissue phantom), and adjust the imaging system to obtain bright signals and a small PSF. Then move to your actual preparation and make further adjustments. Note that biological measurements are not always well served by having the smallest PSF possible. In some cases, slightly underfilling the objective and extending the Z resolution can facilitate measurements, in other cases that can lead to problematic contamination from neuropil or other structures.

Tilt-tip optimization

One of the easier ways to dramatically degrade your imaging quality is to tilt the coverglass relative to the objective. This tilt causes optical aberrations that expand the PSF and result in lower 2p excitation efficiency and resolution. To deal with this, make sure that it is possible to make fine adjustments to the relative tip/tilt of the objective or the coverglass of the preparation. One solution is to mount the preparation on a tip-tilt stage. Note that while many microscopes offer tilting objectives, they typically only tilt around one axis, and thus are not sufficient to compensate for arbitrary tip-tilt.

4.2 Resources

For detailed and practical discussions of building two-photon microscopes, see work by Philbert Tsai and David Kleinfeld [15] and work by Jeff Squire's group [65]. In addition, Labrigger (labrigger.com) is an online resource with a series of technical notes and other information relevant to two-photon imaging.

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Figures

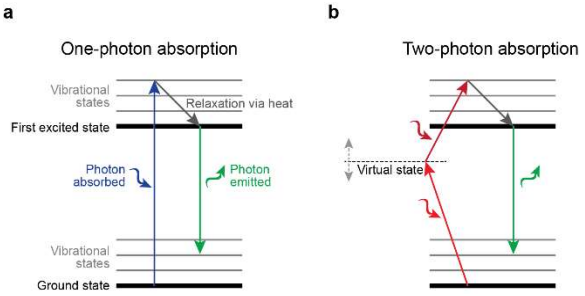

Fig. 1 Energy level (Jablonski) diagram for conventional one-photon excitation and two-photon excitation. (**a**) In one-photon absorption, a high energy (short wavelength, towards the blue end of the visible spectrum) photon is absorbed by a fluorescent molecule which then has a change in electronic structure (is “excited”). The molecule moves from the ground state to the first excited state. The molecule relaxes, via emission of heat, to the lowest vibrational state of the first excited state. Then a lower energy photon (towards the red end of the visible spectrum) is emitted as the molecule relaxes down to the ground state. (**b**) In two-photon absorption, two low energy (near infrared) photons are absorbed nearly simultaneously. A virtual state is drawn on the diagram, but this is not a true energy state for the molecule. The energy level of the virtual state is undefined (unlike true energy states with precise defined energies). Note that the two photons that are absorbed do not have to be of equal wavelength

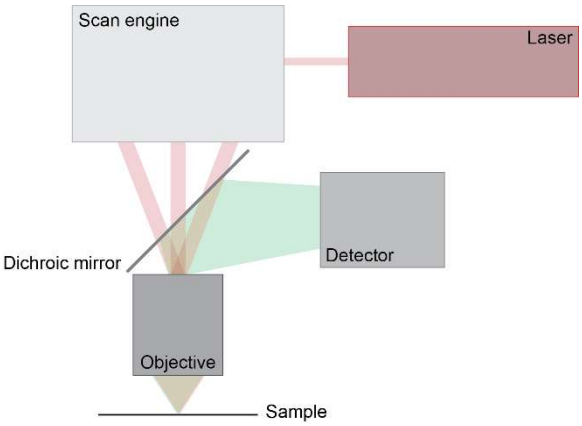

Fig. 2 The basic elements of a two-photon microscope. A block diagram of the key components of a two-photon laser scanning microscope shows that it is essentially simple. The laser beam is scanned such that its center is stationary on the back focal plane of the objective, but its angle of approach varies. The beam should also be expanded to use the full numerical aperture of the objective. Underfilling the objective back aperture will lead to a blurry excitation volume—which leads to both lower resolution and less efficient two photon excitation (due to a lower concentration of photons). The objective performs two jobs: it focuses the excitation light into the sample, and it collects the emitted fluorescence photons. The emitted light is directed to a sensitive detector. The dichroic mirror separates the excitation light and emitted light. This is usually not perfect, and an additional infrared blocking filter in the detection pathway can help prevent excitation light from saturating the sensitive detector